American Journal of Industrial Medicine 22:231–241 (1992)

An Automated Method for the Determination of Acetyl and Pseudo Cholinesterase in Hemolyzed Whole Blood

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The aim of the present study was to develop a method which allows determination of pseudo (PsChE) and acetyl cholinesterase (AChE) activities in single hemolyzed blood samples of workers exposed to cholinesterase-inhibiting compounds, avoiding the time-consuming and laborious separation of plasma and erythrocytes. Two methods based on Ellman's colorimetric determination of cholinesterase activity were compared, and three different substrates were tested.

The best results were obtained with the substrates butyrylthiocholine and acetyl(β-methyl)thiocholine, both showing a substrate specificity of more than 97% with respect to PsChE and AChE, respectively. The method showed sensitivity to detect low levels of inhibition of AChE and PsChE in blood. The between-day precision was less than 4% for both cholinesterase activities. It was demonstrated with this method that hemolyzed blood can be stored at −20°C at least 18 months without loss of cholinesterase activity. The method has been used for 18 months in a monitoring program for laboratory employees working with various cholinesterase-inhibiting compounds. The average coefficients of intraindividual variation amounted to 6.8% (range 2.2–9.6%; 90 percentile, 8%) and 6.6% (range 2.9–9.9%; 90 percentile, 7.9%) for PsChE and AChE, respectively. In a group of non-exposed workers the average intraindividual variations were 4.0% (range 1.5–7.7%; 90 percentile, 7.6%) and 3.6% (range 0.6–6.6%; 90 percentile, 5.3%), respectively. Using the value of 4.0%, it appears possible to detect an individual decrease in cholinesterase activity of more than 8% below a baseline based on three determinations. The method can thus be used to detect relatively low levels of cholinesterase inhibition.

Key words: biological monitoring, carbamate compounds, organophosphorous compounds, colorimetric assay, acetyl cholinesterase, pseudo cholinesterase, pesticide exposures

INTRODUCTION

Cholinesterase inhibition may pose a considerable health risk to workers involved in the production and use of organophosphorous compounds and carbamates. In blood, there are at least two types of cholinesterase: the erythrocyte cholinesterase (AChE; EC 3.1.1.7), also known as acetyl cholinesterase or "true" cholinesterase,

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Accepted for publication November 20, 1991.

which is attached to red blood cells, and plasma cholinesterase (PsChE; EC 3.1.1.8), also known as butyryl cholinesterase or "pseudo" cholinesterase, which is found in plasma and in different organs in the body. Acetyl cholinesterase plays an important role in nerve transmission by hydrolyzing excess acetylcholine at nerve synapses. Pseudocholinesterase has been found in almost all major systems of the body [Trundle and Marcial, 1988], but in neither humans nor animals has a clear biological function been established.

Many carbamates and organophosphorous compounds show acute and chronic effects on the nervous system which are initiated mainly by inhibition of AChE. Clinically manifest symptoms have been reported at an individual level of inhibition of the activity of 50%, while at a level of 80% inhibition, critical symptoms of poisoning have been established [Lewalter, 1986]. The appearance of symptoms depends more upon the rate of fall of cholinesterase activity than on the absolute final level [Trundle and Marcial, 1988]. Although generally the activity of the plasma enzyme decreases more rapidly following exposure than that of the red cell enzyme, the World Health Organization recommends measurement of both enzymes for monitoring occupational exposure to cholinesterase inhibiting compounds [WHO, 1973].

Methods currently in use for measuring cholinesterase activity include electrometric methods [Michel, 1949; Pickering and Martin, 1970], pH-Stat methods [Nabb and Whitfield, 1967], spectrophotometric methods [Augustinsson et al., 1978; Ellman et al., 1961], radiometric methods [Johnson and Russell, 1975], and immunoassay methods [Mehta and Spencer, 1988]. Automated kinetic methods based on Ellman's colorimetric assay have also been reported [Lewis et al., 1981; Hackathorn et al., 1983].

Measurement of both cholinesterase activities requires either separation of plasma and erythrocytes, where the erythrocytes must be washed extensively and hemolyzed prior to measuring acetyl cholinesterase activity, or an inhibitor must be added for one of the enzymes. The aim of the present study was to develop a method with which both cholinesterase activities can be estimated in one hemolyzed whole blood sample without a separation step or addition of an inhibitor. Two methods based on Ellman's colorimetric determination of cholinesterase activity in blood were compared. One of the methods has been described in an epidemiological study by the WHO [1987]. Finally, one of the methods was used for 18 months in routine monitoring of laboratory employees possibly exposed to low levels of cholinesterase inhibiting compounds used in testing protective garments, in chemical synthesis, and in developing analytical methods for detection and disinfection.

MATERIALS AND METHODS

Chemicals

Na_2HPO_4, KH_2PO_4, NaOH, NaCl, saponin, and methanol were purchased from E. Merck, Darmstadt, Germany; acetyl(β-methyl)thiocholineiodide, propionylthiocholineiodide, and ethopropazine HCl were from Sigma Chemical Co., St. Louis, MO, and S-butyrylthiocholineiodide, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB or Ellman's reagent), electric eel acetylcholinesterase (1,000 U/mg), and human pseudo cholinesterase (5 U/mg) from Boehringer Mannheim Co., Mannheim, Germany. Phenyldichlorophosphate was obtained from Aldrich Chemie, Brussels, Belgium. Trans-crotylsarin (trans-2-butenyl-methylphosphonic difluoride) was prepared ac-

cording to the procedure of Bryant et al. [1960] and was a gift of Dr H.P. Benschop, TNO Prins Maurits Laboratory, Rijswijk, The Netherlands. All reagents and chemicals were of analytical grade. Monitrol 1 and 2 control sera were bought from Merz + Dade AG, Düdingen, Switzerland. Solutions were prepared with Millipore® water.

Apparatus

All assays were performed using a discrete kinetic analyzer (ABA-100 bichromatic analyzer, Abbott Laboratories, Pasadena, CA).

Reagents

Phosphate buffer: Method 1. 4.5 g Na_2HPO_4, 0.25 g KH_2PO_4, and 4.5 g NaCL per liter, adjusted with NaOH to pH 8.0; Method 2. 6.1 g Na_2HPO_4 and 2.4 g KH_2PO_4 per liter, adjusted with NaOH to pH 7.2.

Saponin solution: 100 mg saponin was dissolved in 100 ml of the appropriate phosphate buffer by stirring gently.

DTNB reagent: 100 mg DTNB per liter of the appropriate buffer with a final concentration in the assay of 2.4×10^{-4} mol/l.

Inhibitor solutions: Ethopropazine: 32 mg ethopropazine HCl was dissolved in 2 ml of methanol and made up to 50 ml with water. The final concentration in the assay was 2×10^{-5} mol/l. Trans-crotylsarin: Trans-2-butenyl-methylphosphonofluoridate (CRS) (> 98 % pure) was dissolved and diluted in dry isopropanol and stored at −20°C. CRS (25 µl) was added in vitro to 5 ml of heparinized human blood to a final concentration of 12 µmol/l. Phenyldichlorophosphate: Phenyldichlorophosphate (PDP) was dissolved in dry acetonitrile and stored at −20°C. PDP was added in vitro to human blood to a final concentration of 0.6 mmol/l.

Substrates: 75 mg propionylthiocholineiodide (PTCI) in 50 ml water; final concentration in the assay 1×10^{-3} mol/l; 69 mg S-butyrylthiocholineiodide (BTCI) in 1 ml water; final concentration in the assay 7×10^{-3} mol/l; and 47 mg acetyl(β-methyl)thiocholineiodide (ATCI) in 1 ml water; final concentration in the assay 5×10^{-3} mol/l.

Except for the phosphate buffers, all reagents and substrate solutions were prepared daily.

Samples

Blood samples were obtained by finger-tip puncture using lancets or with an automated system (Autolet®; Boehringer Mannheim, Germany). After puncturing with a lancet, 100 µl blood was taken with a calibrated pipette (Sahli, Tamson, Zoetermeer, The Netherlands) and immediately mixed thoroughly with 2.5 ml saponin solution. After puncture with the automated system, 40 µl blood was taken with a calibrated capillary. The capillary was placed in a tube containing 1 ml of saponin solution. The tube was capped and shaken.

All hemolysates were kept at room temperature if the assay was performed within 4 hours; otherwise they were stored at −20°C. The precision of the methods was assessed with commercial control sera (Monitrol 1 and 2). Normally, serum contains only pseudo cholinesterase activity. Therefore, these sera were enriched with purified electric eel acetyl cholinesterase. A pooled hemolyzed blood sample derived from ten subjects was used for long-term quality control.

Assay Procedures

Hemolyzed blood samples were diluted (1:101) with phosphate buffer containing the appropriate substrate and transferred to a multicuvette for the ABA-100. The cuvette was placed on top of the instrument in a water bath kept at 30°C. The absorbance of the colored reaction product (5-thio-2-nitrobenzoic acid) was read using 415/450 nm wavelength filters. The increase in absorbance (E) was followed over three time intervals of 5 minutes and the rate of the reaction (ΔE/min) calculated. ΔE/min is directly proportional to the activity of the enzyme. The ABA-100 is a single beam instrument which operates in a pseudodual beam mode by measuring at two wavelengths, subtracting the absorbance at 450 nm from that at 415 nm. In this manner, cuvette and reagent corrections are handled automatically.

Method 1, a procedure recommended by the WHO [1987], was modified by hemolyzing the blood samples instead of separating erythrocytes and plasma, and adapted for use with the ABA-100. It is based on measurement of both cholinesterase activities with the substrate propionylthiocholine. In the hemolysates, the total cholinesterase activity (total-ChE), i.e., the sum of the activities of acetyl and pseudo cholinesterase, is measured first. After inhibiting PsChE with ethopropazine HCl, the same hemolysates are analyzed for AChE activity. Subtracting the latter result from total-ChE yields the activity of PsChE. Method 2 is the "standard" Ellman assay [Ellman et al., 1961] adapted for use with the ABA-100. S-butyrylthiocholineiodide was used as substrate to determine the activity of PsChE in hemolysates. In a second run with the same hemolysates, acetyl(β-methyl)thiocholineiodide was used as substrate to measure AChE activity.

RESULTS

Specificity

The specificity of substrates for the determination of the two cholinesterases has been discussed for quite some time. For the determination of cholinesterase activity in serum, several substrates have been studied and recommended [Adams and Whittaker, 1949; Heilbronn, 1959; Pilz, 1968]. Butyrylcholine seems accepted as a "specific" substrate for PsChE, and acetyl(β-methyl)choline for AChE [Pilz, 1968]. To investigate the specificity of the substrates in the present methods, solutions of purified PsChE and AChE containing approximately the same activity levels were used. For both methods, the specificity for an enzyme was expressed as the ratio of the enzyme activities measured in the two solutions of AChE and PsChE. By adding ethopropazine to these solutions and to hemolyzed blood samples, it was demonstrated that about 95% of PsChE activity was inhibited while AChE activity remained unaffected. Thus, a high degree of specificity was observed with respect to the determination of AChE and PsChE, with a non-specific reaction being less than 5%. In Table I the results are summarized.

Precision

To establish the within-run precision, samples of Monitrol 1 and Monitrol 2 enriched with electric eel acetyl cholinesterase, and samples of freshly drawn and pooled hemolyzed blood were analyzed six times on 2 days. The between-run precision of the methods was obtained by analyzing the same samples on 6 different days

TABLE I. Assay Specificity Tested With Solutions of Purified Electric eel AChE and Human PsChE

Enzyme assay	Substrate	Activity in solution of AChE U/l	Activity in solution of PsChE U/l	Non-specific reaction (fraction)
Method 1[a]				
Total-ChE	PTCI (A)	4,590	1,815	
AChE	PTCI + I (B)	4,400	65	65/1,750 (=0.037)
PsChE	(A) − (B)	190	1,750	190/4,400 (=0.043)
Method 2				
AChE	ATCI	3,940	65	65/2,800 (=0.023)
PsChE	BTCI	75	2,800	75/3,940 (=0.019)

[a]I, inhibitor; ATCI, acetyl(β-methyl)thiocholineiodide; BTCI, butyrylthiocholineiodide; PTCI, propionylthiocholineiodide.

within 3 weeks. Within-run coefficients of variation (CV) were better than 1.3% for both methods, except for the CV of the PsChE values obtained by subtraction in method 1, which amounted to 4.2%. Between-run CVs are summarized in Table II.

The data show that the CVs of the determination of PsChE via the subtraction procedure of method 1 were much higher than the others. Method 2 yielded relatively low CVs for all samples. Also, the CVs obtained with the hemolyzed blood samples were lower than those of the enriched control sera.

From these results and those on substrate specificity it was concluded that method 2, using ATCI and BTCI as substrates, is to be preferred for monitoring purposes. All further experiments were carried out to validate this method. Hemolyzed blood was chosen for long-term quality control instead of enriched control sera, because the matrix is the same as the actual samples, and in addition, it showed a lower CV. Figure 1 shows an example of a control chart for the PsChE assay. Similar results were obtained for the AChE assay (not shown).

Sensitivity of the Assay

To investigate the sensitivity of the method towards measuring cholinesterase inhibition, experiments were performed using different cholinesterase inhibitors added to human blood samples in vitro. We aimed at obtaining fully inhibited AChE and PsChE activity in blood in vitro without an excess of inhibitor present. Phenyldichlorophosphate (PDP) [Dawson and Poretski, 1988] is a potent cholinesterase inhibitor with a reported rate constant for AChE of 3.5×10^9 l·mol^{-1} min^{-1}. Also, trans-2-butenyl-methylphosphonofluoridate (trans-crotylsarin; CRS) has been described as a potent inhibitor (rate constant: $k_i = 5.5 \times 10^7$ l·mol^{-1} min^{-1} for AChE) [Busker et al., 1991], which is the same order of magnitude as that of soman, a well-known chemical warfare agent. Furthermore, in both cases, the excess amount of added inhibitor is hydrolyzed very rapidly, with reported half-lives for PDP and CRS of 0.15 sec and less than 10 sec, respectively [Dawson and Poretski, 1988; Busker et al., 1991].

The in vitro experiments were performed with freshly drawn human heparinized venous blood which was divided in two portions. To one half, excess amounts of either PDP or CRS were added to final concentrations of 0.6 mmol/l and 12 μmol/l,

TABLE II. Acetyl and Pseudo Cholinesterase Activities and Between-Run Coefficients of Variation (CV) in Various Samples Assayed With Two Methods

Substrate[a]	Enzyme assay	U/l ± s.d. Monitrol 1[b]	Monitrol 2[b]	Hum. blood	Pool blood
Method 1					
PTCI (A)	total-ChE	4,263 ± 327	4,633 ± 283	4,979 ± 278	4,854 ± 313
	CV (%)	7.7	6.1	5.6	6.5
PTCI + I (B)	AChE	2,217 ± 154	2,910 ± 225	3,479 ± 51	3,321 ± 130
	CV (%)	6.9	7.7	1.5	4.1
(A)–(B)	PsChE	2,045 ± 357	1,713 ± 280	1,500 ± 298	1,533 ± 278
	CV (%)	17.5	16.3	19.9	18.3
Method 2					
ATCI	AChE	2,654 ± 171	3,275 ± 278	4,258 ± 99	4,088 ± 98
	CV (%)	6.5	8.5	2.3	2.4
BTCI	PsChE	4,950 ± 307	3,963 ± 308	3,829 ± 110	4,038 ± 156
	CV (%)	6.2	7.8	2.9	3.9

[a]See for abbreviations Table I.
[b]Enriched with electric eel AChE.

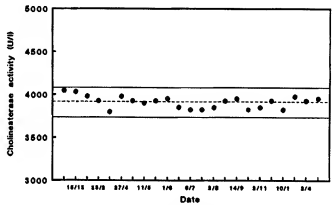

Fig. 1. Quality control chart for the pseudo cholinesterase assay obtained with a hemolyzed pooled blood sample (mean (dashed line) ± 2.5 s.d.).

respectively. The sample was then carefully homogenized and incubated at 37°C for 20 minutes or 3 hours for PDP and CRS, respectively. According to the reported half-lives, these incubation times were amply sufficient to hydrolyze the unreacted inhibitor. To the other half of the blood a corresponding volume of the solvent was added and thereafter the samples were treated similarly. After incubation, mixed samples of different ratios of inhibited and uninhibited blood were constituted, hemolyzed, and assayed to establish the activity of AChE and PsChE. Figure 2 shows a linear relationship between the expected percentage of cholinesterase activity and the measured activity of either AChE or PsChE, of blood inhibited with PDP or CRS. It can be seen from the figure that, although an excess amount of inhibitor was added, still a non-inhibitable fraction remains which was calculated as being, on average, less than 6% for PsChE and AChE.

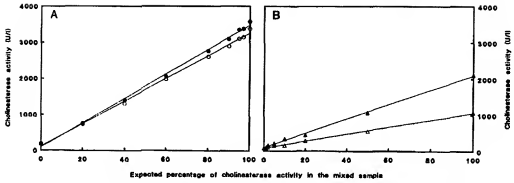

Fig. 2. Activity of AChE and PsChE in mixed human blood samples with different percentages of inhibition. These samples were prepared by mixing parts of uninhibited blood, with parts of blood fully inhibited with either trans-2-butenyl-methylphosphonofluoridate (CRS) or phenyldichlorophosphate (PDP). **A**, Blood inhibited with CRS; (●) PsChE activity; (○) AChE activity; **B**, Blood inhibited with PDP; (▲) PsChE activity; (△) AChE activity.

Stability of Hemolysates

Saponin is a suitable lytic agent, releasing but not inhibiting the membrane-bound enzyme activity [Pickering and Martin, 1970; Demetriou et al., 1974]. There was limited information on the stability of the cholinesterase activity in such hemolysates. To study this, blood samples were obtained from three healthy persons with largely different ratios of PsChE and AChE activities. One half of each sample was divided into 0.5 ml portions, refrigerated immediately, and stored at −20°C (blood samples). The other half was first diluted 26-fold in buffered saponin solution and analyzed immediately in triplicate. The remainder was divided into 0.5 ml portions and refrigerated (hemolysates). During a period of 126 days on 12 different days a stored blood sample and a hemolysate were thawed and analyzed in triplicate. The blood sample was first diluted with freshly prepared buffered saponin. The results for the hemolysates are summarized in Table III.

Hemolysates did not show any loss of activity of PsChE and AChE over a period of 126 days, in contrast to blood samples. In blood that had been stored frozen, the activity of AChE decreased by an average of 13% (range 10–18%) within 21 days, but the PsChE activity remained unchanged. An example of the decrease of acetyl cholinesterase is shown in Figure 3.

Comparison With the Michel Method

A method frequently used in this laboratory and elsewhere is the one described by Michel [Michel, 1949]. To compare Michel's method and method 2, four series of samples, each comprising approximately 50 samples, were assayed. The two methods showed good correlations for PsChE and AChE ($r = 0.98 \pm 0.01$ and 0.88 ± 0.03, respectively). These results are in good agreement with those of Lewis et al. [1981]. Figure 4 shows typical scatter plots.

Linearity of the Enzyme Reaction

The enzyme-substrate reaction for ATCI and BTCI proceeded linearly with time up to at least 45 minutes after addition of the enzyme (approximately 3,000 U/l) to

TABLE III. Effect of Storage at −20°C on Acetyl and Pseudo Cholinesterase Activity of Hemolysates Assayed on 12 Occasions in a Period of 126 Days

	PsChE (U/l ± s.d)	CV (%)	AChE (U/l ± s.d)	CV (%)
Subject 1	756 ± 37	5.0	2,189 ± 65	2.9
Subject 2	2,997 ± 83	2.8	3,600 ± 100	2.8
Subject 3	5,112 ± 138	2.7	3,319 ± 92	2.8

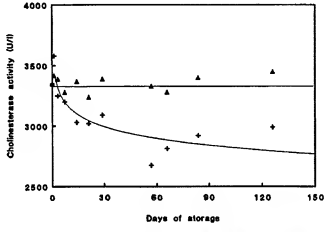

Fig. 3. An example of the effect of storage at −20°C on acetyl cholinesterase activity in hemolysates (▲) and in blood samples (+). The pseudo cholinesterase activity in both types of samples remained unaffected.

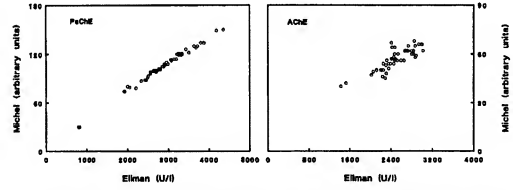

Fig. 4. Scatter plots of enzyme activity in hemolyzed human blood of various workers measured with the pH-method of Michel (ordinate) versus method 2, the colorimetric method of Ellman (abscissa).

the reaction mixture. No lag phase was detected. It was concluded that a reaction time of 15 minutes is adequate to establish enzyme activities up to 9,000 U/l. The limit of detection was about 100 U/l. The in vitro studies with the inhibitors PDP and CRS showed a linear relationship between different percentages of inhibition of either PsChE and AChE activity in blood. In the monitoring program of laboratory technicians, the assay was used successfully for a range of cholinesterase activities of 600–8,000 U/l.

Intraindividual Variation in Cholinesterase Activity

Method 2 was studied in a monitoring program for 106 laboratory technicians involved in research on various cholinesterase inhibiting compounds. Blood samples were obtained at intervals of approximately 5 weeks. It is recommended establishing a personal baseline value with at least three determinations during a period of non-exposure [Coye et al., 1986]. However, it was practically impossible to obtain such baseline values for the workers involved.

To gain insight into the intraindividual variation in cholinesterase activity, 13 non-exposed male laboratory technicians (mean age 38.5 years; sd 13.1, range 27–55) were investigated, taking 3–4 samples from each subject at intervals of 5 weeks. The average intraindividual variations, expressed as CV, were 4.0% (range 1.5–7.7%; 90 percentile, 7.6%) and 3.6% (range 0.6–6.6%; 90 percentile, 5.3%) for PsChE and AChE, respectively. During a period of 18 months (October 1988–April 1990), 1,324 samples derived from a group of 106 laboratory technicians were analyzed.

The data from 17 male workers (mean age 38.8 years, s.d. 9.0, range 29–57) in this group, who were sampled at least ten times, were subjected to a statistical analysis for seasonal trend. No such trend could be detected.

DISCUSSION

AChE and PsChE activities in blood have been determined either after separation of plasma and erythrocytes or by addition of an inhibitor for one of the enzymes. This procedure was considered too laborious for screening workers. Therefore, two methods were investigated for measuring both activities in the same sample after hemolyzing the blood. Using butyrylthiocholine and acetyl(β-methyl)thiocholine as substrates, it was possible to determine both activities in blood samples with an automated method based on Ellman's colorimetric assay. The substrates showed a specificity towards PsChE and AChE of more than 97%, and the between-day precision of the method was less than 4% for both enzymes. In vitro experiments with cholinesterase inhibitors added to human blood showed that the method is sensitive enough to detect low levels of AChE and PsChE inhibition. The non-inhibitable fraction in these blood samples was calculated as being, on average, less than 6% for PsChE and AChE.

The analytical conditions have been chosen so that the reagent and buffer solution are suitable for determination of PsChE and AChE activities over a wide range of activities. The procedure for preparing hemolysates is very simple, and it was demonstrated that there is no loss of activity of the enzymes for at least 126 days when stored at −20°C. A pooled hemolyzed blood sample was used for quality control and appeared to be stable for at least 18 months. Using a semi-automated blood sampling technique (Autolet®) and calibrated capillaries has the advantage of being relatively non-invasive and of drawing accurately small amounts of blood (40 μl). The fact that hemolysates can be stored at −20°C for a long time without loss of activity makes this sampling procedure very useful in field studies where immediate analysis is sometimes impossible.

The intraindividual variation of PsChE and AChE in the general population was reported to be as high as 20–23% and 13–25%, respectively [Hayes, 1982]. The

interindividual variation is even greater. The importance of pre-exposure baseline values for the surveillance of potentially exposed workers is, therefore, readily apparent [Coye et al., 1986]. The square of the intraindividual variation measured (CV_T^2) is the sum of the squared variations arising from blood sampling (CV_s^2), analytical variation (CV_a^2), biological variation (CV_b^2), and the variation of possible exposure (CV_e^2).

The average intraindividual variation in the non-exposed group was relatively low for both enzyme activities: 4.0% (range 1.5–7.7%; 90 percentile, 7.6%) and 3.6% (range 0.6–6.6%; 90 percentile, 5.3%) for PsChE and AChE, respectively. For a group of 64 workers (mean age 37.9 years, s.d. 10.1, range 22–60) sampled at least four times, in the group of 106 workers handling cholinesterase inhibitors, these values were 6.8% (range 2.9–9.9%; 90 percentile, 8%) and 6.6% (range 2.2–9.6%; 90 percentile, 7.9%), respectively. These results are lower than those reported by Hackathorn et al. [1983], i.e., an average of 10% for a non-exposed group and 10.7% (range 9.4–13%) for various exposed groups.

The coefficients of variation for the analytical procedure for the whole validation period were 2.0% for PsChE and 2.8% for AChE. Using these values for the non-exposed group ($CV_e^2 = 0$), a relatively low value for the sum of the biological and sampling variation ($CV_s^2 + CV_b^2$) is obtained. Assuming $CV_s^2 = 0$, the maximum value for the biological variation does not exceed 7.3% and 4.5% for PsChE and AChE, respectively, which is small compared to variations reported in the literature [Hayes, 1982; Hackathorn, 1983].

WHO considers a cholinesterase activity of 30% below the personal baseline level as a biological threshold for withdrawal of the worker from exposure and for implementing preventive actions. This threshold is not derived from medical or epidemiological data but represents the drop from a baseline level based on three determinations in a period of non-exposure which reaches statistical significance [WHO, 1982, 1987]. This threshold reflects levels of precision associated with previous methods for determining baseline values. The average intraindividual variation found for the group of non-exposed workers was small, with a maximum of 7.7%. Following Callaway et al. [1951] and using the average intraindividual variation of 4.0%, it was calculated that a reduction of the cholinesterase activity of more than 8%, below a baseline value based on three determinations, would be statistically significant ($p < 0.05$).

ACKNOWLEDGMENTS

The authors wish to thank Mrs. H.J. Kerstens, Mrs. U. Soekhoe, and Mr. J.C. Ravensberg for skilful technical assistance, Dr. R.W. Busker for his advice in performing the in vitro inhibition experiments and Mrs. M. Saris-Wijnans for statistical analysis. They are grateful to Mr. J.F. Bleichrodt for helpful advice in preparing this manuscript. The valuable discussions with Dr. G. de Mik, Dr. P.C. Bragt, and W.L.A.M. de Kort, MD, are gratefully acknowledged. This work was supported by the Directorate-General of Labor of the Dutch Ministry of Social Affairs and Employment.

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